Developmental Biology **208,** 222–232 (1999)
Article ID dbio.1998.9191, available online at http://www.idealibrary.com on IDEAL®

Limb, Spinal Cord, and Tail Bud Is a Potent Mesoderm Inducer in *Xenopus* Embryos

Laura W. Gamer,[1] Neil M. Wolfman, Anthony J. Celeste, Gary Hattersley, Rod Hewick, and Vicki Rosen

Genetics Institute, Inc., 87 Cambridge Park Drive, Cambridge, Massachusetts 02140

The bone morphogenetic proteins (BMPs) play critical roles in patterning the early embryo and in the development of many organs and tissues. We have identified a new member of this multifunctional gene family, *BMP-11*, which is most closely related to *GDF-8/myostatin*. During mouse embryogenesis, *BMP-11* is first detected at 9.5 dpc in the tail bud with expression becoming stronger as development proceeds. At 10.0 dpc, *BMP-11* is expressed in the distal and posterior region of the limb bud and later localizes to the mesenchyme between the skeletal elements. *BMP-11* is also expressed in the developing nervous system, in the dorsal root ganglia, and dorsal lateral region of the spinal cord. To assess the biological activity of BMP-11, we tested the protein in the *Xenopus* ectodermal explant (animal cap) assay. BMP-11 induced axial mesodermal tissue (muscle and notochord) in a dose-dependent fashion. At higher concentrations, BMP-11 also induced neural tissue. Interestingly, the activin antagonist, follistatin, but not noggin, an antagonist of BMPs 2 and 4, inhibited BMP-11 activity on animal caps. Our data suggest that in *Xenopus* embryos, BMP-11 acts more like activin, inducing dorsal mesoderm and neural tissue, and less like other family members such as BMPs 2, 4, and 7, which are ventralizing and anti-neuralizing signals. Taken together, these data suggest that during vertebrate embryogenesis, BMP-11 plays a unique role in patterning both mesodermal and neural tissues.

Key Words: **BMP; tailbud; limb; spinal cord; *Xenopus*; follistatin.**

INTRODUCTION

Bone morphogenetic proteins (BMPs) were originally discovered based on their ability to induce cartilage and bone formation at ectopic sites in animals (Wozney *et al.*, 1988). As members of the TGF-β superfamily, we know today that BMPs function not only in the skeleton, but also in patterning the early embryo and in the development and differentiation of many organs and tissues. BMPs induce a wide variety of biological responses, including cell proliferation, apoptosis, differentiation, and morphogenesis by signaling through serine-threonine kinase receptors and intracellular effectors known as Smads (reviewed in Hogan, 1996; Whitman, 1998).

Much of our knowledge of the biology of BMP/TGF-β factors comes from analysis of early developmental events in *Xenopus*. For example, in the gastrulating *Xenopus* embryo, different classes of TGF-β superfamily ligands are believed to be involved in patterning of both dorsal and ventral mesoderm. Factors such as activin, Vg-1, and the *Xenopus* nodal-related molecules (Xnrs), have been shown to induce dorsal types of mesoderm such as muscle and notochord (reviewed in Slack, 1994; Harland and Gerhart, 1997). In contrast, BMPs 2, 4, and 7 induce ventral types of mesoderm such as blood (reviewed in Graff, 1997). These BMPs also play a role in patterning ectodermal cells by promoting epidermal differentiation and preventing the formation of neural tissue (Wilson and Hemmati-Brivanlou, 1995). During gastrulation, the dorsal lip or Spemann's organizer of the *Xenopus* embryo provides the critical patterning information described above, to the adjacent mesoderm and overlying ectoderm (reviewed in Harland and Gerhart, 1997; Sasai and DeRobertis, 1997). The activities of the organizer are mediated by a group of secreted molecules that include noggin, chordin, and follistatin, which appear to function as TGF-β/BMP antagonists, blocking ventralizing signals in order to specify dorsal

[1] To whom correspondence and reprint requests should be addressed. Fax: (617) 498-8875. E-mail: lgamer@genetics.com.

0012-1606/99 $30.00

mesoderm and neural tissue (reviewed in Thomsen, 1997). Biochemical studies have shown that noggin and chordin act by binding to BMPs 2 and 4, preventing them from interacting with their receptors (Zimmerman *et al.*, 1996; Piccolo *et al.*, 1996). Follistatin is a potent inhibitor of activin and blocks its signaling by physically sequestering the protein (Nakamura *et al.*, 1990). It has recently been shown that follistatin can also block the activity of BMPs 2, 4, and 7 by forming a trimeric complex with BMP and its receptor (Iemura *et al.*, 1998). These data suggest that during early developmental events, there is not only active patterning by factors like BMPs but also selective inactivation of their signaling pathways by secreted antagonists.

In a search for novel BMP/TGF-β-like molecules, we isolated *BMP-11* by a low stringency screen using a *BMP-7* probe. *BMP-11* is a novel member of the BMP/TGF-β superfamily and is most closely related to *GDF-8/myostatin*, a negative regulator of muscle growth (McPherron *et al.*, 1997). In order to determine the function of *BMP-11*, we characterized its expression pattern during mouse embryogenesis and analyzed BMP-11 protein activity in *Xenopus* embryos. Our data suggest a role for BMP-11 in mesodermal and neural patterning in the vertebrate embryo.

MATERIALS AND METHODS

Isolation of BMP-11. In order to search for novel BMP-like molecules, a probe containing the cysteine-rich mature region (nucleotides 1081–1392) of human *BMP-7* was used to screen a bovine genomic library under reduced stringency conditions (final wash 4× SSC/0.1% SDS at 60°C). Positives from this screen were rescreened with a mixed probe corresponding to the mature regions of human *BMPs 5, 6,* and *7* under high stringency conditions (final wash 0.2× SSC/0.1% SDS at 65°C). Clones which hybridized to the *hBMP7* probe under reduced stringency conditions but not to the h*BMP 5, 6, 7* probe mix at high stringency were further characterized. One of these clones, 7r-30, contained a novel bovine BMP-related sequence designated *BMP-11.* This bovine BMP-related sequence was used to design oligonucleotides, which were used to PCR a human *BMP-11*-specific sequence from a human genomic library (Stratagene, Inc.). The human *BMP-11* sequence was then used as a probe to screen human fetal cDNA and human genomic libraries to derive the full-length sequence of human *BMP-11.* The mature region of human *BMP-11* was then used to screen a mouse genomic library (Stratagene, Inc.) to generate full-length clones and sequence for mouse *BMP-11.* The nucleotide sequences of human *BMP-11* and mouse *BMP-11* have been deposited in the GenBank Sequence database.

Whole mount in situ hybridization. Whole mount *in situ* hybridization was performed as described (Hogan *et al.*, 1994) with minor modifications (Herbert Neuhaus, personal communication). The mouse *BMP-11* probe was a 261-bp fragment derived from the 5′ end of the propeptide region of a *BMP-11* cDNA. Following subcloning into pGEM-3, sense and antisense digoxygenin-labeled riboprobes were produced using SP6 and T7 RNA polymerase.

Whole mount-stained embryos were processed, embedded in paraffin, sectioned at 10 μm, and counterstained with eosin as described (Hogan *et al.*, 1994).

Embryological methods. *Xenopus* eggs were fertilized *in vitro*, and embryos were reared by standard methods (Kay and Peng, 1991). Staging was according to Nieuwkoop and Faber (1967). Animal cap ectoderm was isolated from stage 8–9 blastulae and cultured in 0.5 × MMR and 0.5% bovine serum albumin with or without protein factors until the late gastrula stage. The explants were then transferred to 0.75× NAM for long-term culture. Recombinant human BMP-11, mouse noggin, and human activin A were expressed and produced as previously described (Heine *et al.*, 1987; Schlunegger *et al.*, 1992). Recombinant human FGF-2 was purchased from R&D Systems. Recombinant human follistatin was provided by the NIDDK's National Hormone and Pituitary Program and the NICHD. For histology, animal caps were fixed in Bouin's solution, cleared by 70% ethanol washes, and embedded in paraffin. Explants were serially sectioned at 8 μm and stained with hematoxylin and eosin.

Analysis of RNA by RT-PCR. RNA extraction and RT-PCR analyses were performed as described (Wilson and Melton, 1994) with minor modifications (Amanda Frisch, personal communication). The PCR conditions and sequences of the primers for analysis of Xbra, muscle actin, NCAM, Hox B9, EF-1 α, otx 2, Xtwist, and Mix.1 have been previously described (Hemmati-Brivanlou and Melton, 1994; Wilson and Melton, 1994; Blitz and Cho, 1995; Hopwood *et al.*, 1989; Rosa, 1989).

RESULTS

Cloning of BMP-11

A probe containing the mature region of human *BMP-7* was used to screen a bovine genomic library in order to isolate new BMP-like molecules. Several novel TGF-β/BMP-related sequences were isolated and one was designated *BMP-11.* This bovine *BMP-11* sequence was then used to isolate the human *BMP-11* gene, and mature region sequences from human *BMP-11* were used to clone mouse *BMP-11* (a detailed description of the isolation and characterization of human and mouse *BMP-11* will be described elsewhere). The predicted human BMP-11 protein contains 407 amino acids and displays all the features characteristic of BMP family members including a signal sequence for secretion, an RXXR proteolytic processing site, and a carboxyl terminal region containing the highly conserved pattern of cysteine residues (Fig. 1A). In the C-terminal domain, BMP-11 is more similar to TGF-β and inhibin-β, as it contains nine cysteine residues rather than the seven more commonly seen in BMP proteins. Human and mouse BMP-11 share 99.5% identity over the entire amino acid sequence (data not shown). BMP-11 appears to be most closely related to GDF-8/myostatin, sharing 90% amino acid identity within the carboxyl terminal domain (Fig. 1B). Although BMP-11 is more structurally similar to the inhibin class of TGF-β-related proteins because of its cysteine pattern, it shares equal sequence identity (38–42%) in the mature region to BMP-2 and -4 as it does to inhibin βa, βb, and βc (Fig. 1B).

A

```
MVLAAPLLLGFLLLALELRPRGEAAEGPAAAAAAAAAAAAAAGVGGERSSR  50
PAPSVAPEPDGCPVCVWRQHSRELRLESIKSQILSKLRLKEAPNISREVV  100
KQLLPKAPPLQQILDLHDFQGDALQPEDFLEEDEYHATTETVISMAQETD  150
PAVQTDGSPLCCHFHFSPKVMFTKVLKAQLWVYLRPVPRPATVYLQILRL  200
KPLTGEGTAGGGGGGRRHIRIRSLKIELHSRSGHWQSIDFKQVLHSWFRQ  250
PQSNWGIEINAFDPSGTDLAVTSLGPGAEGLHPFMELRVLENTKRSRRNL  300
GLDCDEHSSESRCCRYPLTVDFEAFGWDWIIAPKRYKANYCSGQCEYMFM  350
QKYPHTHLVQQANPRGSAGPCCTPTKMSPINMLYFNDKQQIIYGKIPGMV  400
VDRCGCS    407
```

B

```
                *         **                                  *   *
BMP-11      CDEHSSESRCCRYPLTVDFEAFGW-DWIIAPKRYKANYCSGQCEYMF---
GDF-8       CDEHSTESRCCRYPLTVDFEAFGW-DWIIAPKRYKANYCSGECEFVF---
BMP-2       ---------CKRHPLYVDFSDVGWNDWIVAPPGYHAFYCHGECPFPLA--
BMP-4       ---------CRRHSLYVDFSDVGWNDWIVAPPGYQAFYCHGDCPFPLA--
Inh-βA      --CDGKVNICCKKQFFVSFKDIGWNDWIIAPSGYHANYCEGECPSHIAGT
Inh-βB      --CDGRTNLCCRQQFFIDFRLIGWNDWIIAPTGYYGNYCEGSCPAYLAGV
Inh-βC      --CQGGSRMCCRQEFFVDFREIGWHDWIIQPEGYAMNFCIGQCPLHIAGM

                                          * *
BMP-11      --MQKYPHTHL-----VQQANPRGSAGPCCTPTKMSPINMLYFNDKQQII
GDF-8       --LQKYPHTHL-----VHQANPRGSAGPCCTPTKMSPINMLYFNGKEQII
BMP-2       DHLNSTNHAIVQTL--VNSVN-SKIPKACCVPTELSAISMLYLDENEKVV
BMP-4       DHLNSTNHAIVQTL--VNSVN-SSIPKACCVPTELSAISMLYLDEYDKVV
Inh-βA      SGSSLSFHSTVINHYRMRGHSPFANLKSCCVPTKLRPMSMLYYDDGQNII
Inh-βB      PGSASSFHTAVVNQYRMRGLNP-GTVNSCCIPTKLSTMSMLYFDDEYNIV
Inh-βC      PGIAASFHTAVLNLLKANTAAGTTGGGSCCVPTARRPLSLLYYDRDSNIV

                      * *
BMP-11      YGKIPGMVVDRCGCS
GDF-8       YGKIPAMVVDRCGCS
BMP-2       LKNYQDMVVEGCGCR
BMP-4       LKNYQEMVVEGCGCR
Inh-βA      KKDIQNMIVEECGCS
Inh-βB      KRDVPNMIVEECGCA
Inh-βC      KTDIPDMVVEACGCS
```

FIG. 1. Sequence of human BMP-11 and its relationship to other members of the TGF-β/BMP superfamily. (A) Predicted amino acid sequence of human BMP-11. The putative proteolytic cleavage site is underlined. The accession number for human BMP-11 is AF100907. (B) Sequence alignment of the carboxyl-terminal region of human BMP-11, mouse GDF-8, human BMP-2, human BMP-4, human inhibin βA, human inhibin βB, and human inhibin βC. Gaps introduced to optimize the alignment are represented by dashes. The conserved cysteine residues are marked by asterisks.

BMP-11 Expression during Mouse Embryogenesis

In order to analyze the spatial expression pattern of *BMP-11*, we performed whole mount *in situ* hybridization on mouse embryos from 8.5 to 12.5 days postcoitum (dpc) and found *BMP-11* transcripts in three main regions: tail bud, limb, and dorsal neural tissue. *BMP-11* was first detected at low levels at 9.0–9.5 dpc in the tip of the tail (Fig. 2A and data not shown). This structure, known as the tail bud, serves as a continuing source of new mesoderm in the postgastrulation embryo (Schoenwolf, 1977; Tam and Beddington, 1987). At 10.0 dpc, expression of *BMP-11* dramatically increases in the tailbud (Fig. 2B). A strong signal continues to be detected in the caudal-most region of the tail until 12.5 dpc (Figs. 2C, 2D, and 2H). Analysis of

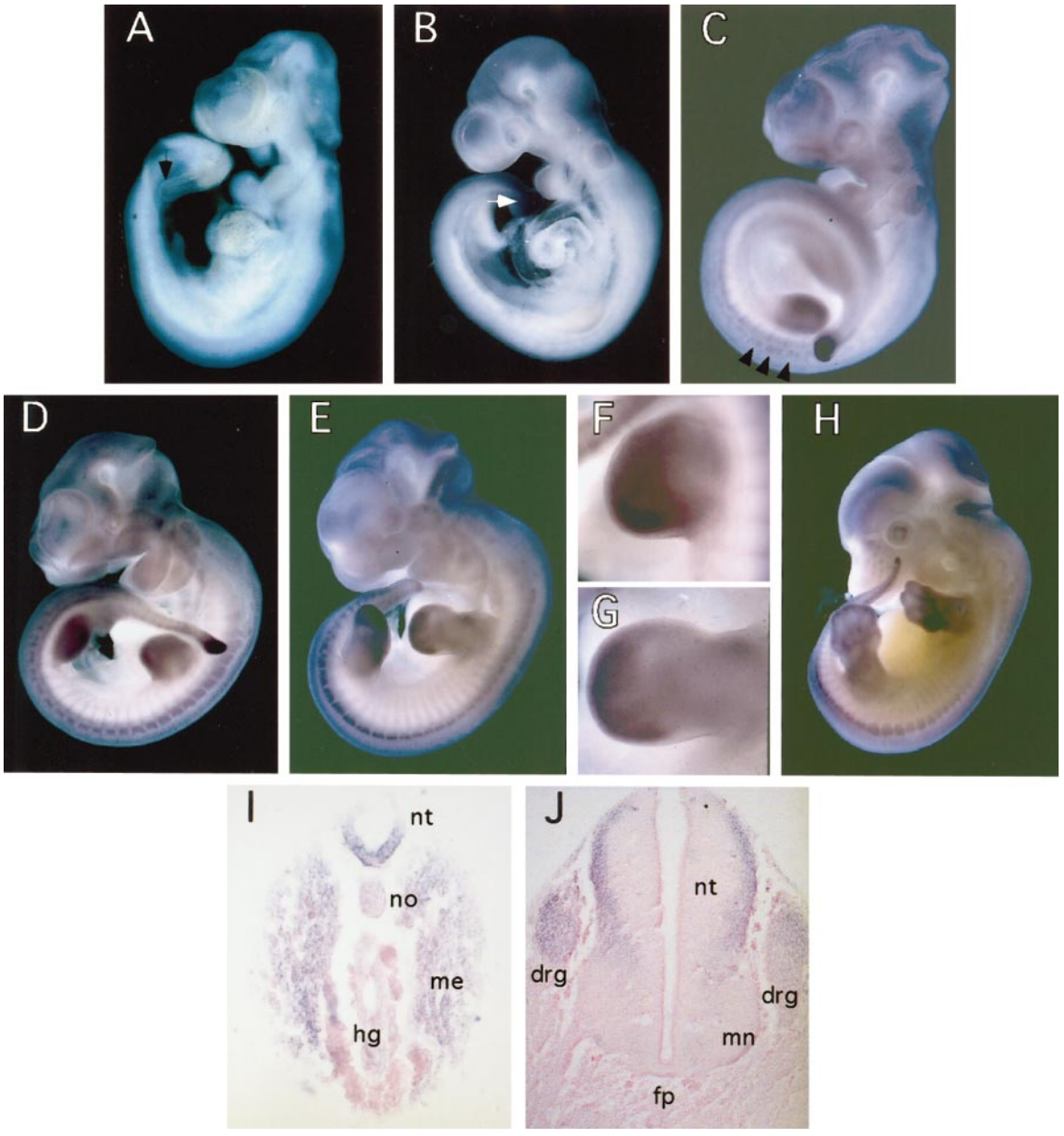

FIG. 2. Whole mount *in situ* hybridization of *BMP-11* expression during mouse embryogenesis. Lateral views of embryos from 9.5 to 12.5 dpc. (A) At 9.5 dpc, weak *BMP-11* expression is seen in the tip of the tail (arrow). (B) At 9.75–10.0 dpc, *BMP-11* expression increases in the tailbud (arrow) and begins to be detected in the developing forelimb bud. (C) At 10.5 dpc, *BMP-11* transcripts are seen in the neural tube and newly differentiated dorsal root ganglia (arrowheads). In the forelimb, *BMP-11* localizes to the distal and posterior mesenchyme. Strong *BMP-11* expression continues in the tailbud. (D) At 11.0 dpc, *BMP-11* expression intensifies in the tailbud, distal limbs, and dorsal root ganglia. (E) At 11.5 dpc, *BMP-11* expression persists in the spinal ganglia, appearing more intense posteriorly, and transcripts in the limbs begin to localize to the mesenchyme surrounding the precartilaginous condensations. (F) High power view of the forelimb at 11.0 dpc showing intense *BMP-11* expression in the distal and posterior regions. No *BMP-11* transcripts are detected in the apical ectodermal ridge. (G) High power view of the forelimb at 11.5 dpc showing BMP-11 expression outlining the newly forming skeletal elements. (H) At 12.5 dpc, *BMP-11* expression is still seen in the spinal ganglia and the caudal most region of the tail. The expression of *BMP-11* in the limb mesenchyme more clearly outlines the forming bones and digits. The apparent signal in the brain, eye, and whisker follicles in whole 11.5–12.5 dpc embryos is due to background associated with older embryos and is seen in the sense strand controls. (I) Transverse section through the tailbud region of 11.0 dpc embryo whole mount *in situ* hybridized with *BMP-11*. Expression is restricted to the neural tube and lateral mesenchyme. (J) Transverse section at a level near the forelimb of an 11.5 dpc embryo whole mount *in situ* hybridized with *BMP-11*. Specific expression is seen in the dorsal lateral region of the developing spinal cord and the dorsal root ganglia. Abbreviations: drg, dorsal root ganglia; fp, floor plate; hg, hindgut; me, mesenchyme; mn, motor neuron; no, notochord; nt, neural tube.

sections through the tail of whole mount-stained embryos at 11.0 dpc reveals specific expression of *BMP-11* in the neuroepithelium and closing neural tube as well as in cells of the dorsal-lateral mesenchyme (Fig. 2I).

The second major area of *BMP-11* expression is in the limbs where expression is initially detected at 10.0 dpc in the distal portion of the forelimb, with the strongest staining in the posterior mesenchyme (Figs. 2B and 2C). From 10.5 to 11.0 dpc, *BMP-11* continues to be expressed at high levels in the distal part of the fore and hind limb, with no signal detected in the apical ectodermal ridge (Figs. 2C, 2D, and 2F). At 11.5 dpc, *BMP-11* expression in the limb mesenchyme begins to outline the precartilaginous condensations, which later differentiate to form bone (Figs. 2E and 2G). *BMP-11* continues to be detected in the mesenchyme between the skeletal elements and is also seen at the digit tips at 12.5 dpc (Fig. 2H).

The third major area of *BMP-11* expression is in the developing nervous system. At 10.5 dpc, *BMP-11* transcripts are detected in the neural tube and the newly forming dorsal root ganglia, which are just beginning to differentiate (Fig. 2C). From 11.0 to 12.5 dpc, *BMP-11* expression is clearly seen in all the dorsal root ganglia, with higher transcript levels detected in the more caudal or newly differentiated spinal primordia (Figs. 2D, 2E, and 2H). Analysis of sections of whole mount embryos at 11.5 dpc revealed specific *BMP-11* expression in the dorsal-lateral edges of the developing spinal cord (excluding the roof plate) and the dorsal root ganglia (Fig. 2J). No expression of *BMP-11* was detected in the ventral neural tube or floor plate. In the dorsal neural tube, the *BMP-11* signal appears to localize to the cell bodies of neurons in the outer mantle layer where sensory relay interneurons develop (Fig. 2J). *In situ* hybridization studies of older embryos (14.0–16.0 dpc) showed that *BMP-11* expression continues in the spinal cord and ganglia, indicating a potential role in later stages of neurogenesis (data not shown).

BMP-11 Induces Morphogenetic Movements in Xenopus Animal Caps

In order to assess the biological activity of BMP-11, we tested the recombinant human protein in the *Xenopus* animal cap assay. Blastula (stage 8) animal pole explants were treated with 200 ng/ml human BMP-11 protein until the gastrula stage (stage 11) and cultured until sibling embryos reached early tailbud tadpole (stage 25). Animal caps cultured in media alone remain round and differentiate into epidermis (Fig. 3A). In contrast, BMP-11 causes a dramatic elongation of animal caps (Fig. 3B). This type of morphology is indicative of mesoderm induction, as the explants try to undergo the movements of gastrulation (Smith *et al.*, 1988). Histological analysis of BMP-11-treated animal caps cultured to late tadpole (stage 38) reveals differentiated blocks of striated muscle, vacuolated notochord cells, and neural tissue (Fig. 3D). Control untreated animal caps form atypical epidermis (Fig. 3C).

Analysis of Gene Expression Induced in Animal Cap Ectoderm by BMP-11

To further assess the kinds of tissues BMP-11 was able to induce, we analyzed gene expression in animal caps treated with various doses of human BMP-11 protein. Animal caps were explanted from late stage 8 embryos and cultured in various concentrations (10 to 1000 ng/ml) of BMP-11 until sibling embryos reached late neurula (stage 22) when they were collected for RT-PCR analysis. We found that BMP-11 induced the expression of the general mesodermal marker, *Xbra*, and the dorsal mesoderm marker, muscle actin, at all doses tested (Fig. 4A). Even at 10 ng/ml (a concentration at which activin, one of the most potent mesoderm inducers, is active), BMP-11 induced *Xbra* and low levels of muscle actin (Fig. 4A). At higher doses (50–1000 ng/ml), BMP-11 induced the pan-neural marker, NCAM (Fig. 4A). This activation of NCAM expression was most likely due to a secondary induction by mesoderm also present in the animal caps (note *Xbra* and muscle actin expression). Our results suggest that in *Xenopus* embryos, BMP-11 is an inducer of dorsal mesoderm and neural tissue.

The mesoderm inducing activity of BMP-11 in the animal cap assay is similar to that of activin but is also comparable to the activity seen for FGF. FGF has been shown to induce mesoderm through a MAP kinase cascade in this system (Gotoh *et al.*, 1995; Umbhauer *et al.*, 1995). Given the recent evidence that BMPs can also activate the MAP kinase pathway (Shibuya *et al.*, 1998), we wanted to determine if BMP-11 was working like activin or FGF to form mesoderm in animal caps. To do this, we treated late blastula stage animal caps with activin (2 ng/ml), FGF (50 ng/ml), and BMP-11 (20 ng/ml) for 2 h (until stage 10.5) and then assayed for the induction of *Mix.1*, a gene that responds to activin, Vg-1, and BMP-4, but not to FGF (Rosa, 1989; Huang *et al.*, 1995; Mead *et al.*, 1996). As expected, activin induced the expression of *Mix.1*, while FGF did not (Fig. 4B). Interestingly, using a relatively low dose of BMP-11, we readily detected the induction of *Mix.1* gene expression (Fig. 4B). These data suggest that in the *Xenopus* embryo, BMP-11 acts more like activin and the TGF-β class of mesoderm inducers.

Follistatin But Not Noggin Inhibits BMP-11 Activity

In *Xenopus* embryos, three secreted factors, noggin, chordin, and follistatin, have been shown to mimic the two main activities of Spemann's organizer: neural induction and dorsalization of mesoderm, by blocking the activities of BMP/TGF-β ligands (reviewed in Harland and Gerhart, 1997; Thomsen, 1997). To determine if any of these antagonists could block BMP-11 activity, we used the animal cap assay. Blastula stage explants were treated with BMP-11 protein (50 ng/ml) alone or with a mixture of BMP-11 (50 ng/ml) and mouse noggin (1000 ng/ml) or human follistatin (500 ng/ml) and cultured until late neurula (stage 22). Noggin, at a 20-fold molar excess, does not block the

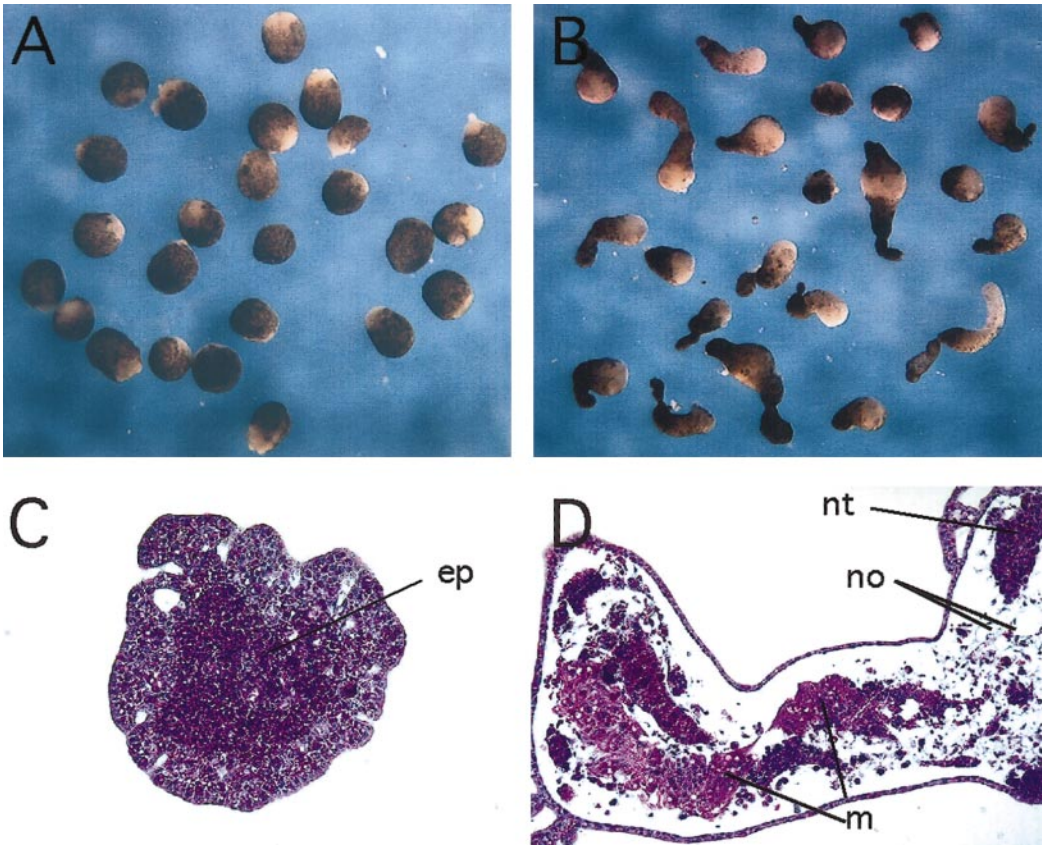

FIG. 3. Morphological and histological analysis of control and BMP-11 treated animal pole explants. Blastula stage (8–9) animal pole explants were treated with 200 ng/ml of recombinant human BMP-11 and cultured to neurula stage for morphology (A, B) and tadpole stage for histology (C, D). (A) Animal caps cultured in media alone remain rounded. (B) Animal caps cultured in BMP-11 protein (200 ng/ml) undergo a dramatic elongation indicative of mesoderm induction. (C) Histological section through a control explant reveals formation of atypical epidermis (ep). (D) Histological section through a BMP-11 treated explant reveals differentiated blocks of muscle (m), notochord (no), and neural tissue (nt).

elongation of animal caps caused by BMP-11, and does not inhibit BMP-11 induction of the dorsal mesoderm marker, muscle actin (Figs. 5B and 5C). The noggin protein used for this assay was active as animal caps incubated in noggin alone at 1000 ng/ml induced the neural marker, NCAM (Fig. 5D). In contrast, follistatin was able to completely inhibit BMP-11-mediated elongation of animal caps at a 10-fold molar excess (Fig. 5F). Moreover, follistatin abolished BMP-11 induction of muscle actin in animal caps (Fig. 5G). In binding experiments, neither noggin nor chordin protein interacted specifically with BMP-11, while follistatin showed significant binding to the BMP-11 protein (L. Fitz and S. Cook, unpublished observations).

BMP-11 Induces Dorsal and Posterior Neural Genes and Modifies the Neural Tissue Induced by Noggin

In *Xenopus*, it is thought that anterior-posterior pattern in the nervous system is induced by the combined action of two signals produced by the dorsal mesoderm (reviewed in Doniach, 1995). The first signal induces anterior neural tissue and can be mimicked by noggin, chordin, and follistatin. The second signal converts the neural tissue induced by the first signal into progressively more posterior types and may reflect the activity of FGFs and Wnts (Cox and Hemmati-Brivanlou, 1995; Lamb and Harland, 1995; McGrew *et al.*, 1995).

At doses from 50 to 1000 ng/ml, BMP-11 induces neural tissue, most likely by a secondary induction through dorsal mesoderm. This raises the question of whether BMP-11 could synergize with neural inducing factors to pattern neural tissue along the anteroposterior axis. In order to address this possibility, we analyzed regional neural marker gene expression in animal cap explants that had been treated with BMP-11 (50 ng/ml), noggin (1000 ng/ml), or a combination of both factors. Animal caps treated with BMP-11 alone induced the expression of the dorsal neural crest marker, *Xtwist* (Hopwood *et al.*, 1989) and the poste-

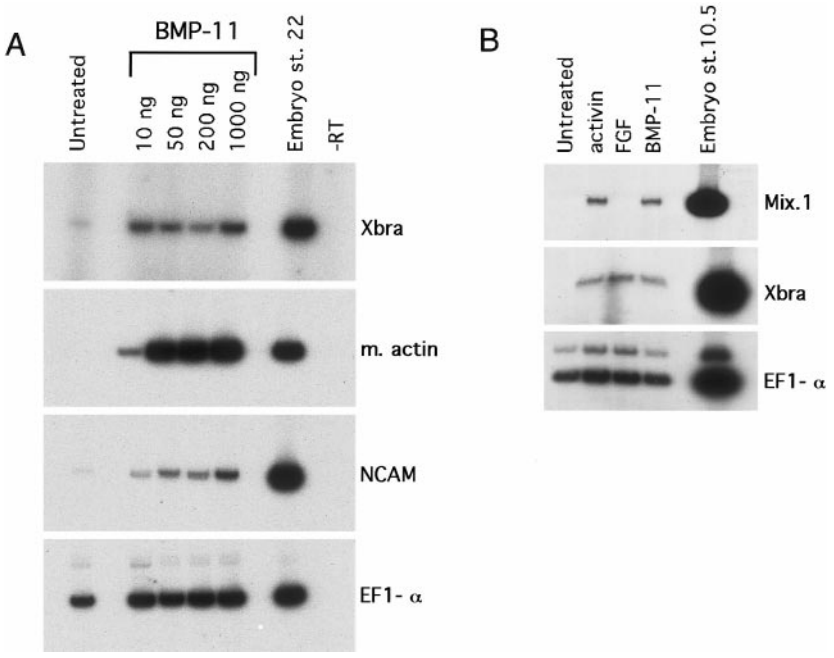

FIG. 4. Analysis of gene expression induced in animal cap explants by BMP-11 protein. (A) Animal caps were explanted from stage 8–9 embryos and treated with increasing doses of recombinant human BMP-11 (10–1000 ng/ml) or buffer alone (untreated) until sibling embryos reached late neurula (stage 22). Total RNA from pools of 10 animal caps was used as the template for cDNA synthesis and the indicated markers were then assayed for by RT-PCR. BMP-11 induced the mesodermal markers *Xbra* and muscle actin (m. actin) at all doses tested. At higher doses (50–1000 ng/ml), BMP-11 induced the pan-neural marker NCAM. EF1-α serves as a loading and reverse transcription control while the embryo and -RT are additional positive and negative controls. (B) Animal caps were explanted from stage 9 embryos and treated for 2 h in buffer alone (untreated), activin (2 ng/ml), FGF (50 ng/ml), or BMP-11 (20 ng/ml) and collected at gastrula (stage 10.5). Low doses of activin and BMP-11 induced the expression of *Mix.1*, while FGF did not. All factors tested induced mesoderm as seen by the expression of *Xbra*.

rior spinal cord marker, *HoxB9* (Wright *et al.*, 1990) (Fig. 6), but not the anterior forebrain marker, *otx2* (Blitz and Cho, 1995). Noggin treated explants only induced anterior neural tissue as detected by *otx2* expression (Lamb *et al.*, 1993) (Fig. 6). Interestingly, animal caps incubated in both BMP-11 and noggin have a reduced level of *otx2* while *Xtwist* and *HoxB9* continue to be expressed (Fig. 6). These results indicate that BMP-11 can modify the neural tissue induced by noggin to more dorsal and posterior fates. This alteration of neural patterning is probably not direct and occurs as a consequence of BMP-11's strong mesoderm-inducing activity.

DISCUSSION

We have been using developmental biology to characterize novel BMP proteins. Here we report the expression pattern and biological activity during vertebrate embryogenesis of *BMP-11*, a new TGF-β superfamily factor that shows high homology to *GDF-8/myostatin*. In the developing mouse, *BMP-11* is expressed in regions of active mesodermal and neural patterning such as the tailbud, limb bud, and dorsal neural tube. In *Xenopus* embryos, BMP-11 is a potent inducer of axial mesoderm (muscle and notochord) and can also induce and modify neural tissue. In addition, BMP-11 activity is inhibited by follistatin but not by noggin or chordin. Taken together our data suggest roles for *BMP-11* in mesodermal formation and neurogenesis in the embryo.

In mouse embryos, *BMP-11* is first detected at low levels in the tip of the tail (tailbud) and as development proceeds, expression dramatically increases and persists until 13.0 dpc. At 9.5 dpc, when we initially see *BMP-11* transcripts in the tail, the tailbud replaces the primitive streak and node as the secondary organizing center of the embryo and new mesoderm continues to arise from this region for several days (Tam and Beddington, 1987; Schoenwolf, 1977). Early and persistent expression of *BMP-11* in the tailbud and its mesoderm inducing ability suggest this factor plays an important role in tail formation and posterior mesodermal patterning. This potential function for *BMP-11* would be similar to that of *Brachyury*, a key regulator of mesodermal cell specification in both mouse and *Xenopus* embryos (Hermann, 1992).

Like many members of the BMP family, *BMP-11* is highly

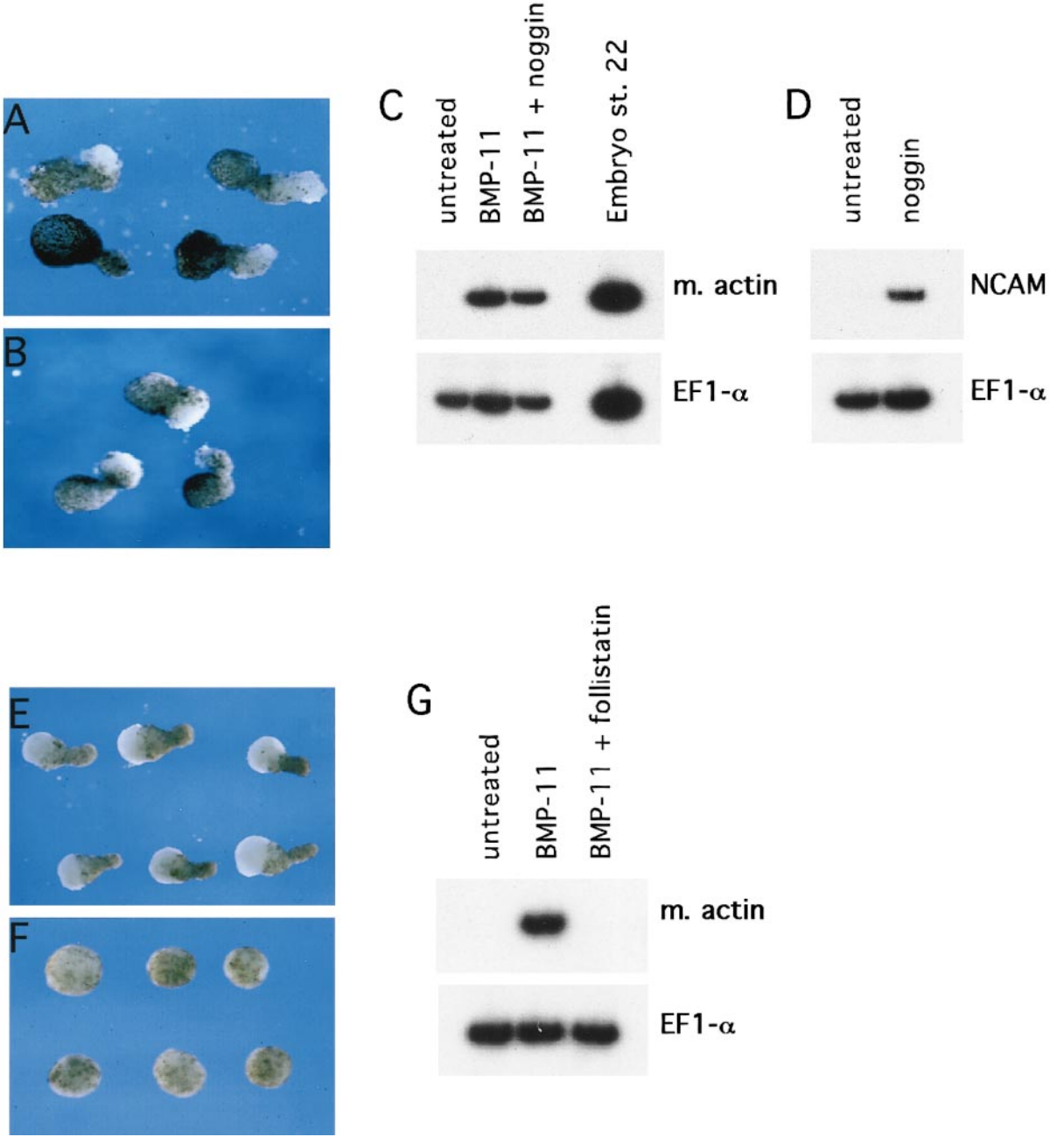

FIG. 5. Follistatin but not noggin inhibits BMP-11 activity. Blastula stage animal pole explants were treated with 50 ng/ml BMP-11 protein alone (A, E) or in combination with 1000 ng/ml mouse noggin protein (B) or 500 ng/ml human follistatin protein (F) until late neurula (stage 22). (A) BMP-11 induces morphogenetic movements in animal caps. (B) A 20-fold molar excess of noggin does not inhibit BMP-11 activity. (C) RT-PCR analysis shows that noggin is unable to block BMP-11 induction of muscle actin (m. actin). (D) Recombinant mouse noggin induces the neural marker, NCAM, indicating the protein has the predicted activity in our assay. (E) BMP-11 protein induces elongation in animal caps. (F) Follistatin completely inhibits the activity of BMP-11 at a 10-fold molar excess. (G) RT-PCR analysis shows that follistatin fully blocks muscle actin induction by BMP-11. EF1-α is a loading and reverse transcription control and untreated animal caps serve as a negative control.

expressed in the developing limb bud, being initially detected in the distal mesenchyme, and later localizing to regions around the developing bones. Initial results from implanting BMP-11 soaked beads in the chick indicate that ectopic BMP-11 causes truncation of skeletal elements in the developing wing (L. Gamer and K. Cox, unpublished

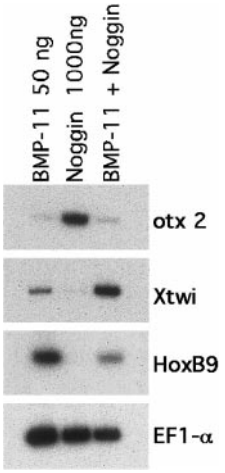

FIG. 6. BMP-11 induces region-specific neural markers and modifies the neural tissue induced by noggin. Blastula stage animal pole explants were treated with BMP-11 (50 ng/ml), noggin (1000 ng/ml), or a combination of both factors until early neurula (stage 18) and analyzed for regional neural marker expression by RT-PCR. BMP-11 induces the neural crest marker, *Xtwist* (Xtwi), and the spinal cord marker, *Hox B9*, but not the forebrain marker, *otx2*. Noggin alone induces only *otx2* expression. In combination, BMP-11 dramatically reduces the amount of *otx2* induced by noggin and allows continued expression of *Xtwi* and *Hox B9*. EF1-α serves as a loading and reverse transcription control.

results). In the context of the limb, *BMP-11* might act as a dual modulator of pattern affecting both mesenchymal cell growth and differentiation. This type of regulatory function is seen with other BMPs such as *BMP-2*, which acts on early undifferentiated chick limb mesenchymal cells to cause apoptosis and then later promotes growth and differentiation of developing cartilage in the limb (Macias *et al.*, 1997).

In the developing nervous system, *BMP-11* is expressed in the neural tube and in the dorsal root ganglia. Dorsal root ganglia are derived from neural crest cells which have already migrated from the dorsal most aspect of the closing neural tube (reviewed in Bronner-Fraser, 1994). Because we do not detect any *BMP-11* expression in this region of the early neural tube, we do not believe *BMP-11* is involved in neural crest cell formation. We do believe, however, that the initial and persistent expression of *BMP-11* in the dorsal root ganglia suggests a later role for this factor in the survival or proliferation of these neurons as they differentiate.

In the neural tube, *BMP-11* localizes to the dorsal lateral edges of the developing spinal cord. Generation of the diverse cell types found in this region of the spinal cord is partially dependent on TGF-β-related signals (reviewed in Tanabe and Jessell, 1996; Bronner-Fraser and Fraser, 1997). For example, *BMP-4* and *BMP-7* are expressed early in the epidermal ectoderm and roof plate and provide dorsalizing signals, which induce neural crest cells and specific subsets of interneurons in developing chick neural tube (Liem *et al.*, 1995, 1997). Because of the later and broader expression of *BMP-11* in dorsal neural tube and its ability to induce and indirectly pattern neural tissue in *Xenopus* embryo explants, we suggest that *BMP-11* may act as a secondary source of BMP signaling, which continues the generation of dorsal cell identity in the developing spinal cord.

The dorsal neural tube is also a rich source of molecules shown to be involved in somitogenesis (for reviews see Currie and Ingham, 1998). In vertebrates, the specification of somitic mesoderm is controlled by factors which are expressed by neighboring tissues. The ventral neural tube and notochord promote formation of the sclerotome, which gives rise to the precursor cells of the ribs, vertebrae, and intervertebral discs. The dorsal neural tube and surface ectoderm promote differentiation of the dermomyotome and myotome, which give rise to dermis and striated muscle. In the chick, BMP-4 has been shown to play a role in dorsal somitic patterning by its ability to block myogenesis in the dermomyotome (Pourquie *et al.*, 1996; Reshef *et al.*, 1998). Based on the above observation, and the expression pattern and activity of *BMP-11*, we suggest that *BMP-11* may also function as a mediator of somite formation, perhaps by modulating sclerotome or dermomyotome differentiation along with other BMPs.

The activity of BMP-11 on *Xenopus* ectodermal explants is most similar to that seen with activin treatment, including induction of dorsal mesoderm and neural tissue. Since BMP-11 is more structurally similar to the inhibin/activin class of TGF-β molecules and because BMP ligands can signal through different receptor combinations (reviewed in Massague, 1998), the activity of BMP-11 could reflect its signaling through an activin type II receptor and downstream activation of the Smad2 pathway. This is most likely the case, as we have found no homologous sequences to *BMP-11* in *Xenopus* by genomic Southern analysis (L. Gamer, unpublished observations). To further investigate this hypothesis, we are currently conducting studies to determine whether the actions of BMP-11 can be blocked by overexpressing dominant negative activin type I and type II receptor constructs in *Xenopus* embryos.

Using the animal cap assay, we also found that BMP-11 activity was specifically blocked by follistatin, but not by noggin. This interaction between BMP-11 and follistatin may be meaningful during development as the expression patterns of both genes localize to adjacent domains in certain regions of the mouse embryo. *BMP-11* is expressed in the dorsal lateral neural tube at a time when follistatin is detected in the adjacent somites (Feijen *et al.*, 1994). In addition, *BMP-11* is expressed around the precartilaginous condensations when follistatin is expressed in those condensing cartilages in the limb (Feijen *et al.*, 1994). Taken together our data suggest that follistatin may be involved in limiting BMP-11 activity during somitogenesis and limb development in a manner similar to the way noggin modifies BMP-4 activity in the somite and limb (McMahon *et al.*, 1998; Reshef *et al.*, 1998; Capdevila and Johnson, 1998).

Recent evidence suggests that follistatin may inhibit BMP activity by a mechanism that is different from noggin and chordin (Iemura *et al.*, 1998). We do not yet know how follistatin antagonizes BMP-11, but binding studies to address this question should be possible upon the identification of a BMP-11 receptor.

BMP-11 is highly related to *GDF-8/myostatin*, a factor recently shown to be an important negative regulator of skeletal muscle mass (McPherron *et al.*, 1997). Interestingly, like other highly related BMPs such as BMP-2 and BMP-4, BMP-11 and GDF-8 proteins appear to have similar activity in the *Xenopus* animal cap assay (L. Gamer, unpublished observations). This suggests that these two factors may be binding to the same or a similar receptor and using an analogous signaling pathway. Although these factors appear to have similar activities, BMP-11 cannot compensate for the loss of GDF-8 in homozygous null animals. This may reflect their mutually exclusive expression domains as *GDF-8* is specifically expressed in developing somites and skeletal muscle (McPherron *et al.*, 1997), where we do not detect any *BMP-11* transcripts. These data suggest that *BMP-11* and *GDF-8* adopted different functions as they evolved from a common ancestral gene.

ACKNOWLEDGMENTS

We thank Amanda Frisch for excellent technical advice with RT-PCR, Diane Peluso for animal cap histology, Jennifer Dube and Adele Pearson for mouse BMP-11 *in situ* probes, and Herbert Neuhaus, Paul Yaworsky, and Lisa-Anne Whittemore for critical reading of the manuscript.

REFERENCES

Blitz, I. L., and Cho, K. W. (1995). Anterior neurectoderm is progressively induced during gastrulation: The role of the *Xenopus* homeobox gene orthodenticle. *Development* **121,** 993–1004.

Bronner-Fraser, M. (1994). Neural crest cell formation and migration in the developing embryo. *FASEB J.* **8,** 699–706.

Bronner-Fraser, M., and Fraser, S. E. (1997). Differentiation of the vertebrate neural tube. *Curr. Opin. Cell Biol.* **9,** 885–891.

Capdevila, J., and Johnson, R. L. (1998). Endogenous and ectopic expression of noggin suggests a conserved mechanism for regulation of BMP function during limb and somite patterning. *Dev. Biol.* **197,** 205–217.

Cox, W. G., and Hemmati-Brivanlou, A. (1995). Caudalization of neural fate by tissue recombination and bFGF. *Development* **121,** 4349–4358.

Currie, P. D., and Ingham, P. W. (1998). The generation and interpretation of positional information within the vertebrate myotome. *Mech. Dev.* **73,** 3–21.

Doniach, T. (1995). Basic FGF as an inducer of anteroposterior neural pattern. *Cell* **83,** 1067–1070.

Feijen, A., Goumans, M. J., and van den Eijnden-van Raaij, A. J. M. (1994). Expression of activin subunits, activin receptors and follistatin in postimplantation mouse embryos suggests specific developmental functions for different activins. *Development* **120,** 3621–3637.

Gotoh, Y., Masuyama, N., Suzuki, A., Ueno, N., and Nishida, E. (1995). Involvement of the MAP kinase cascade in *Xenopus* mesoderm induction. *EMBO J.* **14,** 2491–2498.

Graff, J. M. (1997). Embryonic patterning: To BMP or not to BMP, that is the question. *Cell* **89,** 171–174.

Harland, R. M., and Gerhart, J. C. (1997). Formation and function of Spemann's organizer. *Annu. Rev. Cell Dev. Biol.* **13,** 611–667.

Heine, U. I., Munoz, E. F., Flanders, K. C., Ellingsworth, L. R., Lam, H. Y. P., Thompson, N. L., Roberts, A. B., and Sporn, M. B. (1987). Role of transforming growth factor-β in the development of the mouse embryo. *J. Cell Biol.* **105,** 2861–2876.

Hemmati-Brivanlou, A., and Melton, D. A. (1994). Inhibition of activin receptor signaling promotes neuralization in *Xenopus*. *Cell* **77,** 273–281.

Hermann, B. G. (1992). Action of the Brachyury gene in mouse embryogenesis: Postimplantation development in the mouse. *CIBA Found. Symp.* **165,** 78–91.

Hogan, B., Beddington, R., Costantini, F., and Lacy, E. (1994). "Manipulating the Mouse Embryo," 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Hogan, B. (1996). Bone morphogenetic proteins: Multifunctional regulators of vertebrate development. *Genes Dev.* **10,** 1580–1594.

Hopwood, N. D., Pluck, A., and Gurdon, J. B. (1989). A *Xenopus* mRNA related to *Drosophila twist* is expressed in response to induction in the mesoderm and the neural crest. *Cell* **59,** 893–903.

Huang, H. C., Murtaugh, L. C., Vize, P. D., and Whitman, M. (1995). Identification of a potential regulator of early transcriptional responses to mesoderm inducers in the frog embryo. *EMBO J.* **23,** 5965–5973.

Iemura, S-I., Yamamoto, T. S., Takagi, C., Uchiyama, H., Natsume, T., Shimasaki, S., Sugino, H., and Ueno, N. (1998). Direct binding of follistatin to a complex of bone-morphogenetic protein and its receptor inhibits ventral and epidermal fates in early *Xenopus* embryo. *Proc. Natl. Acad. Sci. USA* **95,** 9337–9342.

Kay, B. K., and Peng, H. B. (Eds.) (1991). *Xenopus* laevis: Practical uses in cell and molecular biology. *In* "Methods Cell Biology." Academic Press, San Diego.

Lamb, T. M., Knecht, A. K., Smith, W. C., Stachel, S. E., Ecomomides, A. N., Stahl, N., Yancopolous, G. D., and Harland, R. M. (1993). Neural induction by the secreted polypeptide noggin. *Science* **262,** 713–718.

Lamb, T. M., and Harland, R. M. (1995). Fibroblast growth factor is a direct neural inducer, which combined with noggin generated anterior-posterior neural pattern. *Development* **121,** 3627–3636.

Liem, K. F., Tremml, G., Roelink, H., and Jessell, T. M. (1995). Dorsal differentiation of neural plate cells induced by BMP-mediated signals from epidermal ectoderm. *Cell* **82,** 969–979.

Liem, K. F., Tremml, G., and Jessell, T. M. (1997). A role for the roof plate and its resident TGFβ related proteins in neuronal patterning in the dorsal spinal cord. *Cell* **91,** 127–138.

Macias, D., Ganan, Y., Sampath, T. K., Piedra, M. E., Ros, M. A., and Hurle, J. M. (1997). Role of BMP-2 and OP-1 (BMP-7) in programmed cell death and skeletogenesis during chick limb development. *Development* **124,** 1109–1117.

Massague, J. (1998). TGFβ signal transduction. *Annu. Rev. Biochem.* **67,** 753–791.

McGrew, L. L., Lai, C-J., and Moon, R. T. (1995). Specification of the anteroposterior neural axis through synergistic interaction of the Wnt signaling cascade with noggin and follistatin. *Dev. Biol.* **172,** 337–342.

McMahon, J. A., Takada, S., Zimmerman, L. B., Fan, C-M., Harland, R. M., and McMahon, A. P. (1998). Noggin-mediated antagonism of BMP signaling is required for growth and patterning of the neural tube and somite. *Genes Dev.* **12,** 1438–1452.

McPherron, A. C., Lawler, A. M., and Lee, S-J. (1997). Regulation of skeletal muscle mass by a new TGF-β superfamily member. *Nature* **387,** 83–90.

Mead, P. E., Brivanlou, I. H., Kelley, C. M., and Zon, L. I. (1996). BMP-4 responsive regulation of dorsal-ventral patterning by the homeobox protein Mix.1. *Nature* **382,** 357–360.

Nakamura, T., Takio, K., Eto, Y., Shibai, H., Titani, K., and Sugina, H. (1990). Activin binding protein from rat ovary is follistatin. *Science* **247,** 836–838.

Nieuwkoop, P. D., and Faber, J. (1967). "Normal Table of *Xenopus laevis*." North-Holland, Amsterdam.

Piccolo, S., Sasai, Y., Lu, B., and DeRobertis, E. M. (1996). Dorsoventral patterning in *Xenopus:* inhibition of ventral signals by direct binding of chordin to BMP-4. *Cell* **86,** 589–598.

Pourquie, O., Fan, C. M., Coltey, M., Hirsinger, E., Watanabe, Y., Breant, C., Francis-West, P., Brickell, P., Tessier-Lavigne, M., and LeDouarin, N. M. (1996). Lateral and axial signals involved in avian somite patterning: A role for BMP-4. *Cell* **84,** 461–471.

Reshef, R., Maroto, M., and Lassar, A. B. (1998). Regulation of dorsal somitic cell fates: BMPs and noggin control the timing and pattern of myogenic regulator expression. *Genes Dev.* **12,** 290–303.

Rosa, F. M. (1989). *Mix.1,* a homeobox mRNA inducible by mesoderm inducers, is expressed mostly in the presumptive endodermal cells of *Xenopus* embryos. *Cell* **57,** 965–974.

Sasai, Y., and DeRobertis, E. M. (1997). Ectodermal patterning in vertebrate embryos. *Dev. Biol.* **182,** 5–20.

Schlunegger, M. P., Cerletti, N., Cox, D. A., McMaster, G. K., Schmitz, A., and Grutter, M. G. (1992). Crystallization and preliminary X-ray analysis of recombinant human transforming growth factor β2. *FEBS Lett.* **303,** 91–93.

Schoenwolf, G. C. (1977). Tail (end) bud contributes to the posterior region of the chick embryo. *J. Exp. Zool.* **201,** 227–246.

Slack, J. M. (1994). Inducing factors in *Xenopus* early embryos. *Curr. Biol.* **4,** 116–126.

Smith, J. C., Yaqoob, M., and Symes, K. (1988). Purification, partial characterization and biological effects of the XTC mesoderm inducing factor. *Development* **103,** 591–600.

Tam, P. P. L., and Beddington, R. S. P. (1987). The formation of mesodermal tissues in the mouse embryo during gastrulation and early organogenesis. *Development* **99,** 109–126.

Tanabe, Y., and Jessell, T. (1997). Diversity and patterning in the developing spinal cord. *Science* **274,** 1115–1122.

Thomsen, G. H. (1997). Antagonism within and around the organizer: BMP inhibitors in vertebrate body patterning. *Trends Genet.* **13,** 209–211.

Umbhauer, M., Marshall, C. J., Mason, C. S., Old, R. W., and Smith, J. C. (1995). Mesoderm induction in *Xenopus* caused by activation of MAP kinase. *Nature* **376,** 58–62.

Whitman, M. (1998). Smads and early developmental signaling by the TGFβ superfamily. *Genes Dev.* **12,** 2445–2462.

Wilson, P. A., and Hemmati-Brivanlou, A. (1995). Induction of epidermis and inhibition of neural fate by BMP-4. *Nature* **376,** 331–333.

Wilson, P. A., and Melton, D. A. (1994). Mesodermal patterning by an inducer gradient depends on secondary cell–cell communication. *Curr. Biol.* **4,** 676–686.

Wozney, J. M., Rosen, V., Celeste, A. J., Mitsock, L. M., Whitters, M. J., Kriz, R. W., Hewick, R. M., and Wang, E. M. (1988). Novel regulators of bone formation: Molecular clones and activities. *Science* **242,** 1528–1534.

Wright, C. V. E., Morita, E. A., Wilkin, D. J., and DeRobertis, E. M. (1990). The *Xenopus* X1Hbox6 homeo protein, a marker of posterior neural induction, is expressed in proliferating neurons. *Development* **109,** 225–234.

Zimmerman, L. B., De Jesus-Escobar, J. M., and Harland, R. M. (1996). The Spemann organizer signal noggin binds and inactivates bone morphogenetic protein 4. *Cell* **86,** 599–606.

Received for publication October 23, 1998
Accepted December 23, 1998